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Importance of the α_3-fragment of complement C4 for the binding with C4b-binding protein

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The human regulatory complement component C4b-binding protein (C4BP) is a multimeric plasma protein, which regulates the classical pathway of the complement system. C4BP functions as a cofactor to factor 1 in the degradation of C4b and accelerates the decay rate of the C4b2a complex. Previously, we have demonstrated that monoclonal antibodies (C4-2 and 9) directed against the α'-chain of C4b inhibit the binding of C4b to C4BP. In order to identify the structural domain of C4b that binds C4BP, proteolytic fragments of C4 were generated with trypsin and *Staphylococcus aureus* V8 protease. Sodium dodecyl sulfate polyacrylamide gel electrophoresis, immunoblotting and amino acid sequence analysis of the proteolytic fragments reactive with the anti-C4 mAb's revealed that the residues Ala^{738}–Arg^{826} of the α_3-fragment of C4b are important for the interaction with C4BP.

Complement regulation; C4b-binding protein; Complement C4

1. INTRODUCTION

Complement C4 is a glycoprotein with a M_r of 189 kDa consisting of three disulfide-linked α (88 kDa), β (72 kDa) and γ (32 kDa) chains [1–6]. Synthesized in the liver as a 185 kDa single chain precursor it undergoes several modifications involving proteolytic excision of two intersubunit-linking peptides [7], sulfatation [8], glycosylation [9], and generation of a thiolester site in the α-chain [10]. The proteolytic cleavage by C1s in the N-terminal part of the α-chain of C4 leads to the generation of the activation peptide C4a (8 kDa) and the activated component C4b (181 kDa). C4b contains at least two binding sites. One is unstable and responsible for the binding of C4b to antibody-Ag aggregates and to acceptor sites on cell surfaces [1–3]. The other binding site of C4b is stable and specific for C2 [4]. C2 bound to C4b will be cleaved by C1s, resulting in the C4b2a complex which acts as the C3 convertase [4].

A regulatory protein of the classical complement pathway is C4b-binding protein (C4BP), a high M_r plasma protein [11–14] which functions as a cofactor to factor 1 in the degradation of C4b and accelerates the decay rate of the C4b2a-complex [13]. In addition, C4BP interacts with one of the plasma proteins in blood coagulation, the vitamin K-dependent protein S [15]. C4BP is composed of multiple identical α-chains with a M_r of 70 kDa and one distinct β-chain with a M_r of 45 kDa that are linked by disulfide bridges [11,16–20]. Each of the 70 kDa subunits forms a tentacle with the N-terminus located at the periphery of the tentacle, whereas the C-termini are located in the central core of C4BP [21–24]. The binding sites for C4b were located on the peripheral half of each tentacle [21,25], whereas one single distinct binding site for protein S was localized near the central core [21,26] on the β-chain [17–20]. The C4b- and heparin-binding function and the factor 1 cofactor activity are located on the 48 kDa chymotryptic fragments [23,25,27,28] of the 70 kDa subunits of C4BP and the region of residues 332–395 seemed important for the binding to C4b [29]. The cleavage of C4b by factor 1 and C4BP in the α'-chain of C4b leads to the generation of the intermediate product, C4b′ after the first cleavage of the α'-chain, resulting in a 70 kDa and a 16 kDa α_4-fragment of the α'-chain both still disulfide linked to the other chains (see Fig. 2). The second cleavage results in the excision of C4d (45 kDa (α_2)), and the generation of a third fragment (27 kDa (α_3)) of the α'-chain still disulfide linked to C4c [14]. In 1984, the primary structure of C4 has been revealed by examining a cDNA coding for C4 [34].

Recently, we have localized the C4BP binding site on the α'-chain of C4b and demonstrated that this binding could be inhibited by mAb's (mAb C4-2 and 9) specific

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Abbreviations: C4BP, C4b-binding protein; mAb, monoclonal antibody; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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for the α'-chain [30]. In this study we identified the epitopes of these mAb's and concluded that the C4BP binding site is localized on the α_3-fragment of C4b.

2. EXPERIMENTAL

2.1. *Materials*

Protein G-Sepharose 4 Fast Flow and a mono Q column were products of Pharmacia LKB Biotechnology Inc. (Uppsala, Sweden). Bovine serum albumin (BSA), Tween-20, 3,3'-diaminobenzidine, dithiothreitol and iodoacetamide were obtained from Sigma Chemical Co. (St. Louis, MO). TPCK-Trypsin and *Staphylococcus aureus* V8 protease were from Worthington Biochemical Corporation, NJ. Prestained molecular weight markers were from Bio-Rad. Polyvinylidene difluoride membranes (Immobilon^{TM}) were from Millipore. All other chemicals obtained were of the best grade available.

2.2. *Purification of proteins*

C4BP was immuno-purified from a barium citrate precipitate of human plasma using anti-C4BP monoclonal affinity chromatography as described [20,28]. Human complement C4 and C4b were obtained as described before [30]. All proteins appeared to be homogeneous as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentrations were determined by measuring the absorbance at 280 nm using an extinction coefficient (1%, 1 cm) of 14.3, 14.1 and 8.7 for the purified mAb's, C4BP and C4, respectively [24,31].

2.3. *mAb's*

mAb's specific for human complement C4 (C4-2, 6, 8 and 9) and C4BP (CORE-6B1) were obtained as described before [28,30]. The murine immunoglobulins were isolated by affinity chromatography on protein G-Sepharose 4 Fast Flow according to the instructions of the manufacturer.

2.4. *Electrophoretic and immunochemical techniques*

SDS-PAGE was performed on 15 or 17.5% slab gels according to Laemmli [32]. After electrophoresis the gels were either stained with Coomassie brilliant blue R-250 or immunoblotted essentially as described by Towbin et al. [33] using a semi-dry electrophoretic transfer apparatus. The Immobilon membranes were either blocked with 5% (w/v) nonfat dry milk in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, incubated with mAb's C4-2, 6, 8 and 9 against the α'-chain of C4b and visualized with peroxidase-conjugated rabbit anti-mouse immunoglobulins or stained with Coomassie brilliant blue.

Alternatively, a C4BP ligand-blotting procedure was used in which a mixture of C4BP (5 μg/ml) and anti-C4BP mAb C4BP-6B1 (5 μg/ml) replaced the primary antibody essentially as described before [30].

2.5. *C4b-C4BP binding assay*

Microtiter plates (strip plate, Costar) were coated overnight at 4°C with C4, C4b or its proteolytic fragments (10 μg/ml in 50 mM $NaHCO_3$, pH 9.6, 50 μl per well). The wells were blocked with 3% (w/v) BSA in 50 mM Tris-HCl, 150 mM NaCl, pH 7.4 at room temperature for 1 h. The wells were washed 5 times with 50 mM Tris-HCl, 150 mM NaCl, pH 7.4 containing 0.2% (v/v) Tween-20 and incubated with varying concentrations of C4BP (0.1–4 μg/ml) and mAb C4BP-6B1 (5 μg/ml) in 3% (w/v) BSA overnight at 4°C. The wells were washed 5 times and the amount of C4BP bound to C4b was detected with peroxidase-conjugated rabbit anti-mouse immunoglobulins.

2.6. *Peptide mapping by SDS-PAGE and immunoblotting*

Reduction of C4 (5.9 mg) was performed at room temperature overnight with 30 mM dithiothreitol in 0.5 M Tris-HCl, pH 8.8 containing 0.05% (v/v) Triton X-100. Alkylation was done at room temperature for 90 min with 50 mM iodoacetamide. Reduced and alkylated C4 was incubated with 3% (w/w) trypsin or 2% (w/w) *S. aureus* V8 protease at 37°C for 2 and 1 h, respectively. The digestion was terminated by the addition of 2 mM DFP (final concentration) on ice. Each digest was separated by SDS-PAGE under reduced conditions and subsequently transferred to an Immobilon membrane. The membranes were incubated with the anti-C4 mAb's and parallel stained with Coomassie brilliant blue R-250. The fragments with positive reactivity with the mAb's were analyzed for N-terminal amino acid sequence.

2.7. *Amino acid sequence analysis*

Amino-terminal amino acid sequence was determined with an Applied Biosystems model 477A gas-phase sequencer using BEGIN-1 and NORMAL-1 cycles, connected to an on-line 120A PTH Analyzer. The proteolytic fragments of C4 transferred to Immobilon membranes were detected by Coomassie staining, excised and placed on polybrene-treated precycled glass fiber filters in the sequencer as described by Matsudaira [35].

3. RESULTS AND DISCUSSION

Our strategy for determining the structural domain(s) of C4 that bind C4BP involved an enzymatical fragmentation of the molecule. Therefore, C4 was incubated with trypsin and *S. aureus* V8 protease at 37°C and subjected to SDS-PAGE under reduced conditions (Fig. 1). SDS-PAGE of the reduced tryptic fragments of C4 showed the liberation of apparent α_2 (45 kDa), α_3 (27 kDa) and α_4 (16 kDa) fragments derived from the α'-chain of C4b, whereas the β- and γ-chains remained rather intact (Fig. 1A, left). Parallel to the liberation of the α_3-fragment, several smaller fragments with apparent M_r of about 23 kDa (T1), 22 kDa (T2), 19 kDa (T3), 10 kDa (T4) and 8 kDa (T5) appeared. Immunoblotting analysis of the tryptic cleavage products of C4 with the mAb's C4-2, 6, 8 and 9 revealed that the mAb's C4-2 and 9 were reactive with the putative α_3-fragment of 27 kDa and the smaller tryptic fragments T1 to T5. The mAb's C4-6 and in less extent C4-8 were reactive with the putative α_2-fragment (Fig. 2A, right). The mAb's C4-2 and 9 had previously been found to inhibit the interaction of C4b with C4BP, whereas the mAb's C4-6 and 8 had no effect [30]. Under non-reduced conditions immunoblotting analysis of the tryptic digest of C4 revealed that the mAb's C4-6 and 8 were reactive with the apparent C4d (or α_2) fragment and that the mAb's C4-2 and 9 were recognizing the C4c fragment with an apparent M_r of 140 kDa (not shown).

To place the tryptic fragments containing a possible C4BP-binding fragment within the structure of the C4 molecule, the N-terminal amino acid sequences of the excised bands were determined. The N-terminal sequences obtained for the fragments T1 to T5 matched that of the N-terminal sequence of the apparent α_3-fragment. It should be remarked that the tryptic fragments T1 and T2 could not completely be excised separately, although this does not effect the sequence results. As representative N-terminal sequence the smallest fragment T5 is indicated in Table I. With the

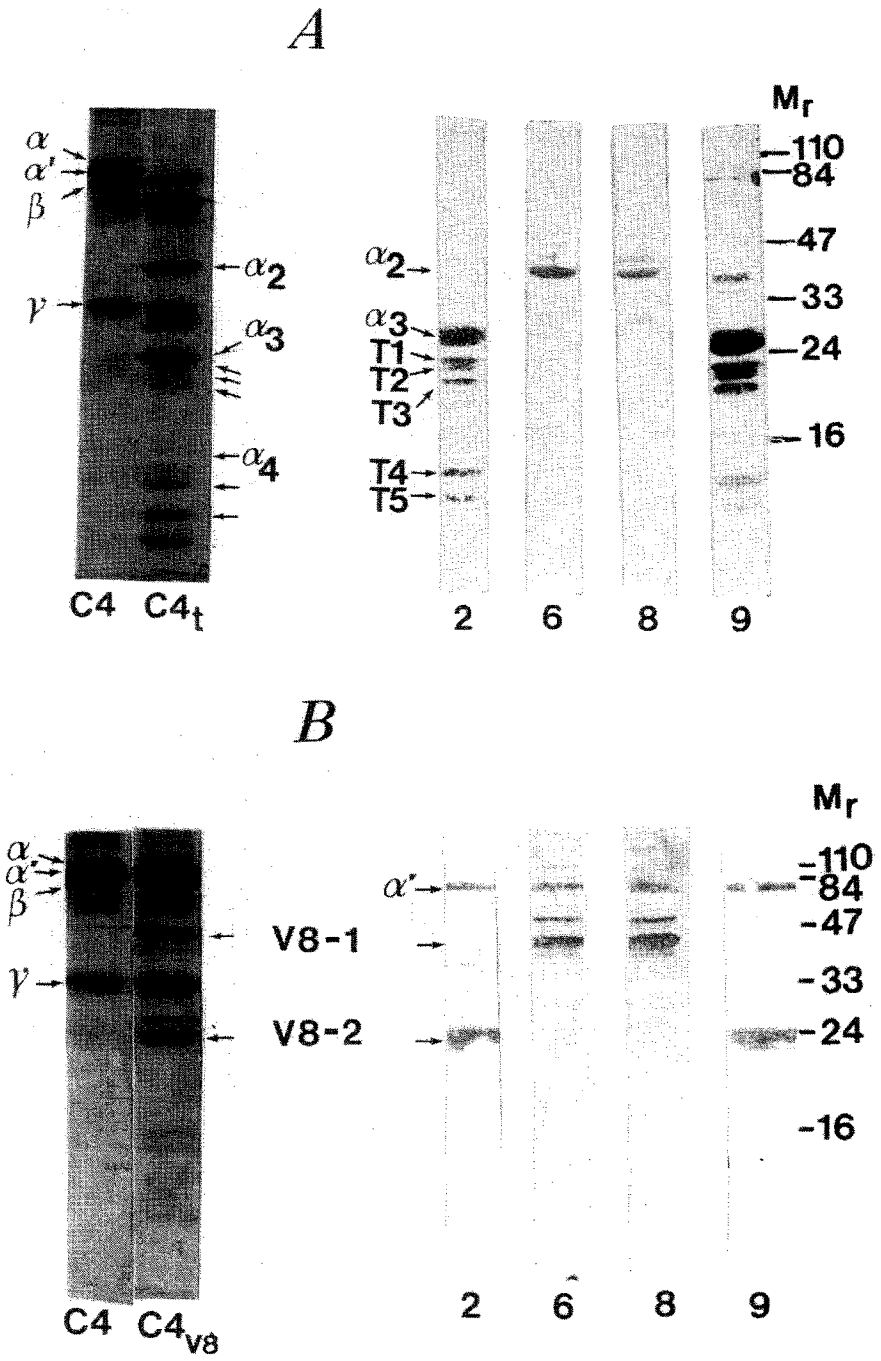

Fig. 1. SDS-PAGE and immunoblotting analysis of proteolytic fragments of C4. Complement C4 was digested with trypsin ($C4_t$; A) and *S. aureus* V8 protease ($C4_{V8}$; B) as described in section 2. The digestion was inhibited by 2 mM DFP dissolved in 5% (v/v) β-mercaptoethanol and subsequently applied on 15% SDS-PAGE. The gels stained with Coomassie brilliant blue are shown to the left, and to the right the fragments transferred to Immobilon membranes, which were analyzed with the mAb's C4-2, 6, 8 and 9 (5 μg/ml) directed against the α'-chain of C4b. In addition, Immobilon membranes were stained with Coomassie brilliant blue from which relevant protein bands were excised from the membrane and directly applied to the cartridge of an Applied Biosystems 477A gas-phase sequencer.

exception of the second residue (which was not determined) this sequence is identical (Table I) to that of the 15 N-terminal amino acids in the α'-chain of C4b [34]. As in 7–15 steps of Edman degradation of the T1/2, T3, T4 and T5 fragments an identical N-terminal sequence was found, it seems that these fragments are derived from the α_3-fragment starting from Ala^{738}. Since an arginine residue is present at position 737, where trypsin would be expected to perform enzymatical cleavage of the molecule and since the ap-

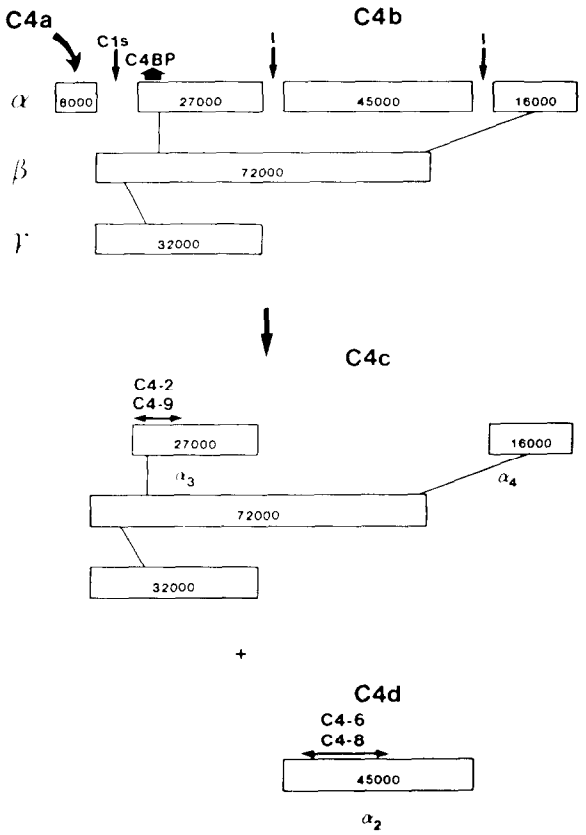

Fig. 2. Schematic representation of C4 and its cleavage fragments. The C1s and factor I (I) cleavage sites are indicated by the arrows. The proposed positions for the epitopes of the different anti-C4 mAb's and the putative C4BP binding site are indicated. Factor I cleaves two peptide bonds in the α'-chain of C4b, giving rise to the C4d (or α_2) fragment (45 kDa), which is released from the remaining part of the molecule (C4c). C4c consists of two α'-chain fragments: an N-terminal α_3-fragment (27 kDa), and a carboxyl-terminal α_4-fragment (16 kDa), both linked to the β- and γ-chain via disulfide bridges.

parent M_r of the T5 fragment is about 8 kDa, it is reasonable to conclude that the domain of C4b recognized by the inhibitory mAb's C4-2 and 9 is identical to the N-terminal domain of the α_3-fragment of C4b, Ala^{738} through Arg^{826}. As a consequence the putative C4BP-binding site is likely composed of the residues Ala^{738}–Arg^{826}. The tryptic fragment α_2, which was recognized by the mAb's C4-6 and 8 was also analyzed for N-terminal sequence. It demonstrated the N-terminal sequence indicated in Table II. This sequence is identical to that of the first eleven amino acids (i.e., residues 961–972) after the Arg^{960}–Val^{961} cleavage site, indicating that the epitopes for the mAb's C4-6 and 8 are located on the α_2 (or C4d) fragment. As our apparent α_2-fragment seems to start from Val^{961}, it is likely that this fragment is a smaller derivative of the commonly accepted α_2 (or C4d) fragment starting from Thr^{938} [34]. No smaller tryptic fragments derived from the C4d-fragment were recognized by the mAb's C4-6 and 8.

Trypsin-treated C4b did not demonstrate any C4BP binding activity in either the C4BP ligand blotting procedure or the C4b-C4BP binding assay, while untreated C4BP gave good binding. Also the binding of intact C4BP to the C4b coated microtiter wells was not inhibited by the addition of the proteolytically cleaved C4b digest.

When C4b was digested with *S. aureus* V8 protease and examined under reduced conditions on SDS-PAGE, the α'-chain was also predominantly shown to be cleaved into a 44 kDa (V8-1) fragment and a 23 kDa (V8-2) fragment (Fig. 1B), whereas the β- and γ-chains remained intact. Immunoblotting analysis of *S. aureus* V8 protease-treated C4b demonstrated that the mAb's C4-6 and 8 were reactive with the α'-chain and the V8-1 fragment, whereas the mAb's C4-2 and 9 recognized the α'-chain and the fragment V8-2. The N-terminal sequence obtained for fragment V8-2 matched that of the N-terminus of the α'-chain of C4b starting from Ala^{738}, indicating that the epitopes of the mAb's C4-2

Table I

Sequence analysis of the tryptic C4 fragment T5

Cycle no.	PTH	N-terminal sequence[a]
1	A	A
2	ND[b]	L
3	E	E
4	I	I
5	L	L
6	Q	Q
7	E	E
8	E	E
9	D	D
10	L	L
11	I	I
12	D	D
13	E	E
14	D	D
15	D	D

[a] N-terminal amino acid sequence according to Belt et al. [34].
[b] ND, not determined.

Table II

Sequence analysis of the tryptic α_2-fragment of C4

Cycle no.	PTH	N-terminal sequence[a]
1	V	V
2	T	T
3	A	A
4	S	S
5	D	D
6	P	P
7	L	L
8	D	D
9	T	T
10	L	L
11	G	G

[a] N-terminal amino acid sequence according to Belt et al. [34].

and 9 are located at the N-terminal end of the α'-chain of C4b (Table III). No further sequence studies were done with fragment V8-1. The *S. aureus* V8 protease C4 fragments V8-1 and 2 did not demonstrate any C4BP binding abilities. To characterize the epitopes of the mAb's C4-2 and 9 and thus the putative C4BP-binding domain in more detail, we attempted to prepare lower M_r fragments by further proteolysis of the tryptic fragments of C4 with *S. aureus* V8 protease. Although indeed lower M_r fragments were obtained by tryptic and subsequent V8 protease cleavage, these fragments were no longer reactive with the mAb's.

Recently, we reported [30] that the C4BP-binding site on C4b was located on the α'-chain of C4b and because of the observation of binding of C4BP to a 70 kDa band in reduced C4b, which was likely to be the partly degraded α'-chain of C4b' [36], we excluded the α_4-fragment as a putative C4BP-binding fragment. And because C4c still demonstrated a low affinity for C4BP [37], in contrast with C4d, it was suggested that the binding site for C4BP was located at the N-terminal α_3-fragment of C4b and that possibly the negatively charged region between the residues 743 and 752 was important for the interaction with C4BP [30]. Utilizing our mAb's C4-2 and 9 against the α'-chain of C4b which had previously been found to inhibit the binding of C4BP to C4b we have now been able to identify a 88 residue sequence with the help of the published sequence of the molecule [34] at the N-terminal end of the α_3-fragment of the C4b molecule that is involved in the interaction of C4b with C4BP (Fig. 2). Our experiments also suggest that both the tertiary structure of C4b and the linear sequence of amino acids in the α_3-fragment are important for a proper interaction of C4b with C4BP. This is demonstrated by the absence of any C4BP binding abilities of the α_3-fragment of C4b alone. Our experiments do not rule out the possibility that other sites (conformational or structural) in the α'-chain of C4b are also important in its interaction with C4BP.

Previously, we have demonstrated that C4BP binds both to C4b and to the negatively charged heparin via its N-terminal 48 kDa chymotryptic tentacles, which have been shown to contain basic residues between short consensus repeats 1 and 2 of the α-chains of C4BP [28,29,38]. The C4BP binding region we have identified in this study contains a cluster of 7 acidic amino acids between the residues 744 and 752 in C4b that could be important for binding. This consideration is consistent with a previous study in which was demonstrated that the affinity of C4b for C4BP was enhanced under reduced ionic strength [37]. However, the importance of this negative domain alone could not be demonstrated with a corresponding synthetic peptide, because this peptide failed to react with the anti-C4 mAb's and more important did not effect the binding of C4BP to C4b (data not shown). In conclusion, the present observations allow a tentative assignment of the C4BP-binding site to the residues Ala^{738}–Arg^{826} of the 771 amino acid long α-chain of C4 (Fig. 2), but a possible involvement of the negatively charged amino acids in the region Glu^{744}–Asp^{752} remains to be determined.

Table III

Sequence analysis of the *S. aureus* V8 protease C4 fragment V8-2

Cycle no.	PTH	N-terminal sequence[a]
1	A	A
2	L	L
3	E	E
4	I	I
5	L	L
6	Q	Q
7	E	E

[a] N-terminal amino acid sequence according to Belt et al. [34].

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